Cloning of Tumor-associated Differentially Expressed Gene-14, a Novel Serine Protease Overexpressed by Ovarian Carcinoma


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ABSTRACT

The family of enzymes known as serine proteases supports many biological functions for cancer cells, including activation of growth and angiogenic factors and activation of other proteases for invasion and metastasis. In addition, many of these serine proteases are secreted by cells into the extracellular space to serve these functions. Therefore, serine proteases are excellent candidate tumor markers. To examine serine proteases expressed by ovarian carcinoma, we designed degenerate PCR primers corresponding to the conserved regions of these genes and used them in reverse transcriptase-PCR experiments with normal and tumor cDNA as a template. The PCR products were cloned and sequenced, and one of these clones was found to encode a novel serine protease, named tumor-associated differentially expressed gene-14 (TADG14). Northern blot analysis indicated that the mRNA for TADG14 is 1.4 kb long and that it is highly overexpressed in ovarian carcinoma compared with normal ovary. The entire cDNA has been obtained, and based on sequence homology, it encodes a 260-amino acid serine protease. Semi-quantitative PCR indicates that TADG14 is overexpressed in 24 of 40 tumors studied. Northern blot data confirm this overexpression, and immunohistochemical staining suggests that this protein is secreted. As such, the TADG14 protease may be useful as a diagnostic tool or as a molecular target for therapy.

INTRODUCTION

Serine proteases comprise a family of protein-degrading enzymes that serve as a host of biological functions, including activation of blood coagulation cascades, activation of growth and angiogenic factors, and degradation of extracellular matrix components (1–4). In recent years, aberrant expression of serine proteases such as plasminogen activator has been shown to correlate positively with the invasiveness and metastatic potential of tumor cells (3, 5, 6). Presumably, this occurs because the ability of the tumors to degrade extracellular matrix components is increased, either directly or indirectly through the proteolytic activation of other zymogenic proteases. More significantly, the serine protease known as PSA has been used successfully as a tumor marker for the early diagnosis of prostate cancer due to its abnormal prevalence in the peripheral blood of these patients (7). Serine proteases play important roles in the cascade of events involved in the malignant process, and at least for prostate cancer, they provide sufficient signal to allow detection of early disease.

This year, the American Cancer Society predicts that there will be 14,500 ovarian cancer-related deaths in the United States and that there will be 25,400 new cases diagnosed (8). Unfortunately, although diagnostic assays based on the ovarian cancer antigen CA125 have improved physicians’ ability to diagnose and monitor recurrence of ovarian carcinoma, the problem remains that most of these new cases will be diagnosed at late stages, in which the primary tumor has progressed to a metastatic state. This failure to diagnose patients in the early stages of ovarian cancer directly impacts the 5-year survival rate for ovarian cancer patients, which remains below 50% (8). This study is the result of a strategy to identify those serine proteases that are overexpressed by ovarian carcinomas in an effort to define potential tumor markers.

All serine proteases contain conserved histidine, aspartate, and serine residues that are necessary for enzymatic activity. To identify the expressed serine proteases, we used degenerate oligodeoxynucleotide primers designed to the conserved amino acid sequences surrounding the invariant His and Ser residues of the catalytic triad (9) in PCRs with cDNA from either normal ovarian tissue or ovarian carcinoma as the template. PCR products of the appropriate size were subcloned into T-vector and sequenced. Previously, this strategy has proved successful in identifying the serine proteases hepsin and stratum corneum chymotryptic enzyme, which have been shown to be expressed at abnormally high levels in ovarian carcinoma (10). Homology searches revealed that one of the subclones obtained from ovarian carcinoma represented a novel 406-bp sequence that has significant sequence similarity to other known proteases, including mouse neuropsin, hHk2, and human PSA. The complete cDNA for this novel sequence was cloned and found to encode a tryps in like serine protease, named TADG14. More importantly, the TADG14 transcript was found to be highly expressed in a majority of ovarian tumors but not expressed by normal ovarian tissue. High-level expression of TADG14 appears to be restricted to tumors, and this protease appears to be secreted in a manner that would suggest a possible role in invasion and metastasis. Moreover, due to the extracellular nature of this enzyme, it may be possible to exploit its expression as a diagnostic tool for ovarian cancer.

MATERIALS AND METHODS

mRNA Isolation and cDNA Synthesis. Forty-one ovarian tumors and 10 normal ovary specimens were obtained from surgery and frozen in liquid nitrogen or purchased from the Cooperative Human Tissue Network. mRNA isolation was performed according to the manufacturer’s instructions using the oligo(dT) chromatography-based Mini RiboSep Ultra mRNA isolation kit (Becton Dickinson, Bedford, MA).

First strand cDNA was synthesized using 5.0 μg of mRNA and either random hexamer or oligo(dT) primers according to the manufacturer’s protocol using a first strand synthesis kit (Clontech, Palo Alto, CA). The purity of the cDNA was evaluated by PCR using primers specific for the p53 gene. These primers span an intron such that the PCR products generated from pure cDNA can be distinguished from cDNA that is contaminated with genomic DNA.

PCRs. Reactions with degenerate primers and quantitative PCRs were carried out as described previously (10). The sequences of the TADG14-
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Fig. 1. Northern blot analysis. A, mRNA was isolated from the tissues of interest as described (10) and subjected to Northern hybridization using a random-labeled 230-bp TADG14-specific RT-PCR product. The blot was stripped and probed for B- tubulin. B–D, MTN blots (Clontech) were probed with the same TADG14- and β-tubulin-specific RT-PCR products. TADG14 mRNA was detected as a 1.4-kb transcript in tumors and was not detected in any normal tissue studied.

Fig. 2. cDNA and deduced amino acid sequences of TADG14 and comparison of predicted TADG14 sequence with known proteases. A, the cDNA sequence of TADG14 is shown with its deduced 260-amino acid sequence represented by the one-letter code for each residue. Within the cDNA, the Kozak’s consensus sequence (ACCATGG) can be predicted TADG14 sequence with known proteases. The boxed and circled portions represent the polyadenylation signal and the stop codon, respectively. B, using the Genetics Computer Group Pileup program, the amino acid sequence of TADG14 was compared with hHk2 (accession no. P06870), human PSA (hPSA, accession no. P07288), mouse neuropsin (mNeur, accession no. P07288), and human protease M (bProM, accession no. U62801). Black shading, amino acid residues that are identical in at least three of the five sequences. Gray shading, amino acids that are similar among at least three sequences. ▲, positions of the residues of the catalytic triad.
buffer (pH 6.0). The specimens were incubated in methanol with 0.3% H2O2 stained with ethidium bromide. In this figure, the 454-bp band represents the experiment. The reaction products were electrophoresed through a 2% agarose gel and products were quantitated as described (10).

negative controls were performed by using normal serum instead of the primary antibody.

RESULTS

After confirming that the 406-bp PCR product was unique and was appropriately conserved to fit into the serine protease family, we used this PCR product as a probe for Northern blot analysis to determine the transcript size and tissue specificity of its expression. It was found that the mRNA for this clone is ~1.4 kb (Fig. 1A) and that it is strongly expressed in ovarian carcinomas but not in normal ovary. More importantly, the transcript was found to be undetectable by Northern analysis in 28 normal human tissues studied (Fig. 1, B–D; data not shown). In a more sensitive assay of 50 normal human tissues (Clontech), RNA dot blot analysis revealed that this clone was very weakly expressed in only 3 of these 50 tissues: the kidney, the lung, and the mammary gland (data not shown).

Using standard hybridization techniques, we screened a cDNA library that was constructed from the mRNA isolated from an ovarian cystadenocarcinoma patient. Five clones were obtained, two of which overlapped and spanned 1343 nucleotides (Fig. 2A). The last two nucleotides prior to the poly(A) tail and the poly(A) tail itself were obtained from the National Center for Biotechnology Information EST database (accession no. AA343629). Subsequent Northern blot analyses with probes derived from sequences near the 5’ or 3’ end of this cDNA were consistent with previous results suggesting that the obtained clones were produced by the same gene (data not shown). This cDNA includes a Kozak’s consensus sequence for the initiation of translation and a polyadenylation signal. The mRNA provides an open reading frame of 260 amino acids, which contains the necessary residues (His73, Asp120, and Ser212) in the appropriate context to classify this protein as a trypsin-like serine protease (11). Near its NH2 terminus, the predicted protein contains a stretch of hydrophobic amino acids that probably serve as a secretion signal sequence (12). In addition, residues 110–112 encode a potential site for glycosylation that is common to serine proteases of the kallikrein subfamily, such as PSA. This enzyme was named TADG14, and the sequence was submitted to GenBank (accession no. AF055982).

Comparison of the deduced TADG14 amino acid sequence with sequences of known proteases revealed that it possesses significant similarity with hHk2, PSA, protease M, and mouse neuropsin (13–16). At the amino acid level, TADG14 is 48% identical to protease M,

Immunohistochemistry. Immunohistochemical staining was performed using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Formalin-fixed and paraffin-embedded specimens were routinely deparaffinized and processed using microwave heat treatment in 0.01 M sodium citrate buffer (pH 6.0). The specimens were incubated in methanol with 0.3% H2O2 for 30 min at room temperature and then incubated with normal goat serum for 30 min. The samples were incubated with anti-TADG14 peptide-derived polyclonal antibody for 1 h at room temperature in a moisture chamber, followed by incubation with biotinylated antirabbit IgG for 30 min, and then incubated with ABC reagent (Vector Laboratories) for 30 min. The final products were visualized using the AEC substrate system (DAKO, Carpenteria, CA), and sections were counterstained with hematoxylin before mounting. Negative controls were performed by using normal serum instead of the primary antibody.

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To characterize the extent and frequency of expression of the TADG14 gene in ovarian tumors, we used semiquantitative PCR with cDNA derived from normal ovary, ovarian carcinoma, or LMP tumors as template. This technique has been previously authenticated and verified by Northern blot, Western blot, and immunohistochemistry (10, 18). PCR primers that amplify a TADG14-specific 230-bp product were synthesized and used simultaneously in reactions with primers that produce a specific 454-bp PCR product for β-tubulin. A radiolabeled nucleotide was included in this reaction, the PCR products were separated on a 2% agarose gel, and the intensity of each band was quantitated by a PhosphoImager (Molecular Dynamics).

Immunostaining for the mouse protease TADG14 was performed with the TADG14-1 antibody for normal ovary (A), two serous carcinomas (B and C), mucinous carcinoma (D), endometrioid carcinoma (E), and clear cell carcinoma (F) of the ovary. No staining was observed in normal ovary. The serous carcinoma shown in B has TADG14 most strongly associated with the surface of the tumor, whereas in the serous tumor in C, TADG14 is found in a granular form in an apparent secretion pathway. In mucinous carcinoma, TADG14 appears to be most highly expressed along the invasive front of the tumor. TADG14 is secreted into the lumen of the glandular structure formed by the endometrioid carcinoma in E. The clear cell carcinoma stained in F shows diffuse staining throughout all tumor cells.

The ratio of the TADG14 PCR product to that of β-tubulin (mean ± SD) was calculated for normal ovary (0.034 ± 0.024) samples, which all showed relatively low expression levels. TADG14 overexpression was defined as exceeding the mean of the ratio of TADG14 to β-tubulin for normal samples by >2 SDs. TADG14 was found to be overexpressed in 4 of 10 LMP tumors (40%) and 20 of 30 ovarian carcinomas (67%) studied. For individual histological subtypes of tumor, the expression ratio was 0.110 ± 0.092 for serous LMP tumors, 0.096 ± 0.142 for mucinous LMP tumors, 0.457 ± 0.345 for serous carcinomas, 0.171 ± 0.300 for mucinous carcinomas, 0.308 ± 0.144 for clear cell carcinomas, and 0.485 ± 0.325 for endometrioid carcinomas. Of the 30 ovarian carcinomas studied, 13 of 17 serous tumors, 1 of 7 mucinous tumors, 3 of 3 clear cell tumors, and 3 of 3 endometrioid tumors overexpressed TADG14 (Fig. 3B). These data are summarized in Table 1. Although not quantitated, transcripts for TADG14 were detectable in prostate and colon carcinoma (data not shown).

Immunogenic poly-lysine-linked multiple-antigen peptides were synthesized based on the deduced amino acid sequence of TADG14 and used to immunize rabbits for the production of polyclonal antibodies. The antisera raised to the peptide sequence LDWIKKIG-SKG near the COOH terminal (amino acids 249–260) was used in Western blot analysis to determine whether this antibody would recognize a protein of the predicted size of M, 28,000. Proteins from the HeLa cell line and the carcinoma-derived MD-MBA-435S cell line were used in this experiment, and it was found that the antibody recognized a single M, 30,000 protein in both (Fig. 4, two right lanes). This size is within a reasonable range of the predicted molecular weight. As a negative control, duplicate HeLa and MD-MB435S lysates were examined with rabbit preimmune serum (Fig. 4, left two lanes). More importantly, this experiment was reproducible with antisera to a peptide from a different region of TADG14, suggesting that these cultured cancer cells produce the TADG14 protein.

Immunohistochemical staining supported the data obtained by quantitative PCR and by Northern blot. Using a TADG14 peptide-directed antibody (T14-1; described above), we observed no staining with normal ovarian tissue samples (Fig. 5A). However, intense staining was associated with tumor cells of all of the various histological subtypes of ovarian carcinoma examined. For serous carcinoma (Fig. 5, B and C), the antigen appears to be associated with tumor cells in the form of granules. These granular structures may be intermediates in the pathway that ultimately leads to secretion of TADG14. In mucinous and clear cell carcinoma samples (Fig. 5, D and F, respectively), TADG14 is highly associated with the tumor cells. In endometrioid carcinoma (Fig. 5E), the antigen is most prevalent in the glandular lumen formed by the tumor cells.

**DISCUSSION**

The lethality of neoplastic cells lies in their ability to proliferate abnormally and invade normal host tissues. Malignancies use proteases to provide a variety of services that assist in the process of tumor progression, including activation of growth and angiogenic factors, and to provide the basis for invasion and metastasis. In the process of studying these enzymes, we have identified overexpression of the known proteases, hepsin and stratum corneum chymotryptic enzyme. In this study, we have cloned a cDNA encoding a novel serine protease, TADG14. This protease was found to be highly expressed in 67% (20 of 30) of ovarian carcinomas studied, whereas...
it was undetected in normal ovarian tissue. We were also unable to
detect the TADG14 transcript in any of 50 normal human tissues
studied. Upon prolonged Northern blot exposure, extremely low lev-
els of TADG14 were detected in normal kidney, breast, and lung. This
suggests the possibility that this gene is under the control of a
promoter that is most active in ovarian tumors, and it may be possible
to exploit this for therapeutic means. Unfortunately, TADG14 expres-
sion can be detected in other types of cancer, including prostate,
breast, and colon. This may limit the usefulness of TADG14 as a
potential diagnostic maker for ovarian carcinoma, but it in no way
detracts from the usefulness of this molecule as a target for cancer
therapy or the usefulness of the TADG14 promoter in gene therapy
applications.

At the nucleotide level, TADG14 mRNA resembles the recently
cloned human neuropsin transcript, with obvious differences residing
in the 5′ and 3′ UTRs. TADG14 mRNA contains 491 bases of 5′ UTR
that were not found in human neuropsin. Also, the nucleotides pre-
ceding the poly(A) tail in the 3′ UTR are not homologous. A 0.9-kb
transcript for human neuropsin was identified in cultured keratino-
cytes but not in normal hippocampus. Also, it was not identified as
being associated with tumors. At the amino acid level, TADG14 is
identical to human neuropsin.

Among other known proteases, TADG14 most closely resembles
the mouse protease known as neuropsin, which was originally cloned
from mouse hippocampus and, subsequently, implicated in neuronal
plasticity (19). If TADG14 functions in a manner similar to mouse
neuropsin, it may be capable of restructuring the three-dimensional
architecture of a tumor, allowing for shedding of tumor cells or
invasion of normal host tissues by degrading fibronectin (20). In
support of this, immunohistochemical staining of ovarian tumors
suggests that TADG14 is highly associated with tumor cells and the
cells near the invasive fronts of tumor. Therefore, TADG14 could be
an important target for the inhibition of tumor progression.

Most importantly, the 5-year survival rate for ovarian cancer pa-
ients remains <50% because of an inability to diagnose this disease
at an early stage. TADG14 contains a secretion signal sequence and
immunohistochemical data suggest that TADG14 is secreted. In
addition, by Northern blot and RNA dot blot analyses, TADG14 appears
only in abundance in tumor tissues. As a result of this, it may be
possible to design assays based on the detection of this protein for the
early detection of ovarian cancer. Currently, the best available ovarian
cancer tumor marker is CA125. However, due to high endogenous
circulating levels of this antigen, the signal:noise ratio limits its
usefulness as a diagnostic tool. Therefore, TADG14, due to its limited
expression in other tissues and potential for being present in the
circulation of tumor-bearing patients, may prove to be a useful tool for
early detection of ovarian cancer, especially the most prevalent serous
cystadenocarcinoma subtype.

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